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Cardiac fibroblasts require focal adhesion kinase for normal proliferation and migration

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Manso AM, Kang SM, Plotnikov SV, Thievessen I, Oh J, Beggs HE, Ross RS. Cardiac fibroblasts require focal adhesion kinase for normal proliferation and migration. *Am J Physiol Heart Circ Physiol* 296: H627–H638, 2009. First published January 9, 2009; doi:10.1152/ajpheart.00444.2008.—Migration and proliferation of cardiac fibroblasts (CFs) play an important role in the myocardial remodeling process. While many factors have been identified that regulate CF growth and migration, less is known about the signaling mechanisms involved in these processes. Here, we utilized Cre-LoxP technology to obtain focal adhesion kinase (FAK)-deficient adult mouse CFs and studied how FAK functioned in modulating cell adhesion, proliferation, and migration of these cells. Treatment of $FAK^{flox/flox}$ CFs with Ad/Cre virus caused over 70% reduction of FAK protein levels within a cell population. FAK-deficient CFs showed no changes in focal adhesions, cell morphology, or protein expression levels of vinculin, talin, or paxillin; proline-rich tyrosine kinase 2 (Pyk2) expression and activity were increased. Knockdown of FAK protein in CFs increased PDGF-BB-induced proliferation, while it reduced PDGF-BB-induced migration. Adhesion to fibronectin was not altered. To distinguish between the function of FAK and Pyk2, FAK function was inhibited via adenoviral-mediated overexpression of the natural FAK inhibitor FAK-related nonkinase (FRNK). Ad/FRNK had no effect on Pyk2 expression, inhibited the PDGF-BB-induced migration, but did not change the PDGF-BB-induced proliferation. FAK deficiency had only modest effects on increasing PDGF-BB activation of p38 and JNK MAPKs, with no alteration in the ERK response vs. control cells. These results demonstrate that FAK is required for the PDGF-BB-induced migratory response of adult mouse CFs and suggest that FAK could play an essential role in the wound-healing response that occurs in numerous cardiac pathologies.

extracellular matrix; cytoskeleton; fibroblast

THE MAIN CELLULAR COMPONENTS of the heart include cardiac myocytes, fibroblasts (CFs), endothelial cells, and smooth muscle cells, with the majority being myocytes and fibroblasts. Early studies in rat myocardium established that CFs comprised over 70% of the total number of cardiac cells (35, 63). A more recent study established that the percentage of CFs in the heart varies between species, being a higher number in larger mammals and lower one in small mammals, such as mice, in which CFs represent ~30% of the total cells in the heart (1). Under basal conditions, CFs contribute to extracellular matrix (ECM) deposition and create a scaffold for cardiac myocytes (2, 7). CFs are the main source of interstitial collagen deposition in the normal and pathological heart. They maintain ECM integrity and myocardial structure (11, 12). In cardiovascular pathologies, such as hypertensive heart disease, or ischemic and diabetic cardiomyopathy, fibroblasts play an important role in the myocardial remodeling process (61). With remodeling, hypertrophy of cardiac myocytes may occur, but alterations in CF number, location, migration, and proliferation can also be seen, leading to changes in the amount and composition of the cardiac ECM. Excessive fibroblast proliferation or increase in ECM content can lead to cardiac fibrosis and increased myocardial stiffness and can cause cardiac dysfunction (6). Furthermore, fibrosis can lead to myocyte hypoxia, via perivascular scarring, or disrupt cell-cell communication between myocytes, predisposing to arrhythmias (46). Wound healing is an essential response after myocardial infarction, and CFs are the main cellular component in this process, migrating to the ischemic/peri-infarct area, increasing synthesis of ECM proteins to replace necrotic myocytes and then forming a scar (54, 61). Although cardiac fibrosis is clearly associated with altered myocardial mechanical performance, arrythmogenesis, and wound-healing responses, the function of CFs is still poorly understood, particularly compared with cardiac myocytes. Furthermore, while substances such as growth factors and cytokines, or mechanical stress (2, 7), have been shown to regulate CF growth and migration, less is known about the signaling mechanisms involved in these processes. Recent data suggest that interactions between the ECM and integrins, and signaling events that can arise from this interaction, are linked to these responses. Integrins are heterodimeric cell-surface receptors that link the ECM and the intracellular cytoskeleton. They serve both as adhesive receptors and also direct intracellular events (45). In binding of cells to ECM, focal adhesion (FAs) are formed, and it is at these sites that a large number of proteins are recruited to form a signaling complex.

FA kinase (FAK) is a nonreceptor protein-tyrosine kinase that is one component of the FA complex that plays a major role in signaling pathways initiated by integrins (33). FAK also participates in signal transduction by G-protein-coupled receptors, such as angiotensin II, and receptor tyrosine kinases, such as the platelet-derived growth factor (PDGF) receptor (47). In

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various cell types, FAK may function as a convergence point for signaling pathways triggered by integrins and other humoral factors that are important in the regulation of cell function (33).

Several recent studies have shown the important role for FAK in the myocardium. For example, FAK expression and activity were increased in the volume-overloaded myocardium of patients with chronic mitral regurgitation (30). These changes were mainly related to ones in CFs, as opposed to myocytes, and they paralleled the increase in interstitial fibrosis seen in those patients. Thus FAK may contribute to the fibrogenesis and structural abnormalities found in chronic volume overload of the heart (30). Moreover, small interfering RNA knockdown of FAK expression in all cells within the intact mouse heart allowed for reduced fibrosis in response to chronic pressure overload (9). The pressure-loaded FAK-deficient mice had improved heart function vs. control animals, indicating the important role of fibrosis in the remodeling process following pressure overload.

Despite this information, little data exist on the function of FAK in adult CFs. In this study, we utilized a genetically modified mouse model to obtain FAK-deficient adult CFs and studied how FAK functioned in modulating cell adhesion, proliferation, and migration of these cells. Our results demonstrate that FAK is required in the migration of CFs as a response to growth factors and suggest that FAK is involved in the fibrotic process found in numerous cardiac pathologies.

MATERIALS AND METHODS

Antibodies and reagents. Mouse monoclonal antibodies and their sources were as follows: vinculin and talin (clone hVIN and 8D4, respectively) both from Sigma (St. Louis, MO); α-smooth muscle actin and GAPDH (clone 1A4 and 6C5), both from Santa Cruz Biotechnologies (Santa Cruz, CA); and paxillin (clone 349, BD) from Transduction Laboratories (San Jose, CA). Rabbit polyclonal antibodies and their sources were as follows: FAK NH_2 terminus (A-17), FAK COOH terminus (C-20), desmin (H-76), and discoidin domain receptor 2 (H-108) from Santa Cruz Biotechnologies; proline-rich tyrosine kinase 2 (Pyk2) from Upstate/Millipore (Lake Placid, NY); phospho-FAK Y397 and phospho-Pyk2 Y402 from Biosource International (Camarrillo, CA); phospho-ERK and phospho-JNK from Stressgene (Victoria, Canada); ERK1/2, p38, phospho-p38, and JNK from Cell Signaling (Danvers, MA); 4,6-diamidino-2-phenylindole dihydrochloride hydrate from Sigma (St. Louis, MO); and peroxidase-conjugate AffiniPure Donkey anti-rabbit antibody from Jackson ImmunoResearch Laboratories (West Grove, PA). PDGF-BB was from Biosource (Carlsbad, CA). Alexa Fluor 488 goat anti-mouse, Alexa Fluor 568 goat anti-rabbit secondary antibodies, and phalloidin were from Molecular Probes/Invitrogen (Carlsbad, CA).

Animals and preparation of adult mouse CFs. Construction of the "floxed" FAK allele and production of genetically manipulated mice was as published previously (3). These mice contain loxP sites flanking exon 18, the kinase domain of the FAK gene. All animals were housed in an American Association for Accreditation of Laboratory Animal Care approved facility. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the Veterans Administration San Diego Healthcare System. Primary adult mouse CFs were isolated from hearts of homozygous "floxed" mice (termed $FAK^{flox/flox}$) that were maintained in a C57BL/6 background, by using previously published methods (59). The cells were grown to confluence at 37°C with 95% air/5% CO_2 in growth media [DMEM, 10% bovine calf serum (BCS) (Hyclone), with the addition of 1% penicillin and 1% streptomycin (PS) (Invitrogen)]. Cultures consistently showed fibroblast morphology by microscopy and were positive for the discoidin domain receptor 2, a marker for fibroblasts (34), and negative for desmin, a smooth muscle cell marker (data not shown). All cells were used between *passages 2* and *4*.

FAK-deficient CFs were created by adenoviral infection of the homozygous floxed CFs derived from the FAK-flox mice ($FAK^{flox/flox}$), with a Cre-recombinase-expressing adenovirus (Ad/Cre) (Fig. 1*A*). CFs were seeded at a concentration of 15×10^4 cells/well onto six-well plastic plates in media containing DMEM, 10% BCS, and 1% PS. They were rendered quiescent by their exposure to a defined serum-free DMEM containing 0.1% bovine serum albumin (BSA) + 1% PS for 24 h before infection. For virus infection, CFs were incubated with adenoviruses Ad/Cre [200 multiplicity of infection (MOI)] or Ad/FRNK (25 MOI) for 96 and 48 h, respectively, as outlined in RESULTS. Stimulation with PDGF-BB (10 ng/ml) was subsequently performed. Control cells for all studies were created by infection of the $FAK^{flox/flox}$ CFs with an adenovirus expressing β-galactosidase (Ad/LacZ) at matched MOIs and times. FAK gene excision and FAK protein reduction were evaluated with polymerase chain reaction (3), Western blot analysis, and immunofluorescent staining, respectively.

Western blotting. At study termination, cells were washed in cold PBS and scraped into lysis buffer (150 mM $NaCO_3$, 1 mM EDTA, pH = 11) containing protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Equal amounts of protein lysate were separated by SDS-PAGE using 9% polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) by electroblotting. Membranes were blocked in PBS containing Tween (0.1%), as well as 5% nonfat dry milk, and incubated with the primary antibodies overnight at 4°C. Bound antibodies were visualized by using secondary antibodies with horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) and ECL reagents (Amersham Pharmacia Biosciences/GE Healthcare, Piscataway, NJ).

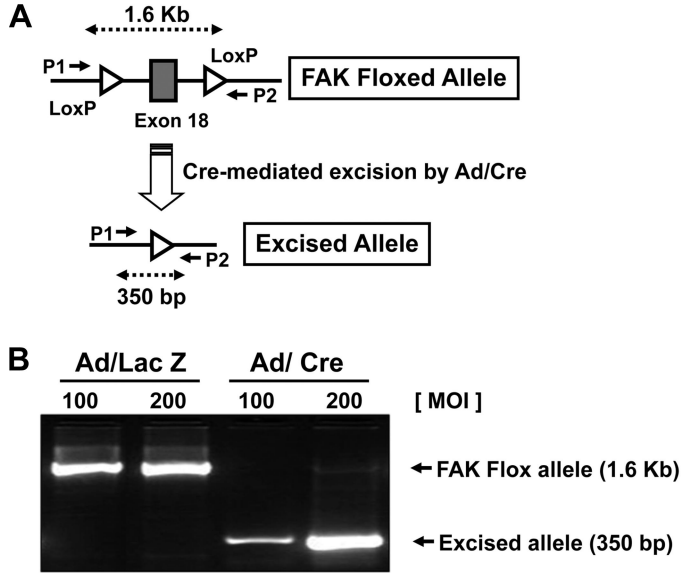


Fig. 1. Excision of the focal adhesion kinase (FAK) gene in $FAK^{flox/flox}$ cardiac fibroblasts (CFs) by recombinant adenoviral Cre-recombinase virus (Ad/Cre). *A*: diagrams of FAK floxed and excised alleles. Primers used for identification of the unexcised and excised alleles are P1 and P2 (note: diagrams are not drawn to scale). *B*: PCR fragments from intact or excised floxed alleles were detected at 1.6 kb or 350 bp, respectively. The 350-bp excised fragment was detected in $FAK^{flox/flox}$ CFs infected with Ad/Cre virus at both 100 and 200 multiplicity of infection (MOI). No excised fragment was detected in $FAK^{flox/flox}$ CFs infected with adenovirus expressing β-galactosidase (Ad/LacZ) (control) virus.

Immunofluorescent staining and FA analysis. Cellular immunostaining was performed as described previously (39). Briefly, 96 h after infection, CFs were trypsinized and resuspended in DMEM supplemented with 0.1% BSA and 1% PS and plated for 16 h on glass coverslips coated with fibronectin (FN) at a concentration of 10 μg/ml; fixed with 4% paraformaldehyde (PFA), permeabilized in 0.2% Triton X-100/PBS, and blocked with 3% BSA and 1.5% goat serum in PBS. Primary antibodies [FAK (1:100), Pyk2 (1:100), vinculin (1:400), and paxillin (1:200)] were applied overnight at 4°C. After washing, secondary antibodies were applied [Alexa-488 or Alexa-568 (1:500)] for 30 min at room temperature. Microscopic analysis was performed using a Zeiss Standard microscope equipped with epifluorescent optics and photographed using a Hamamatsu cooled charge-coupled device camera (Hamamatsu Photonics, Bridgewater, NJ). Quantitative analysis of FA size and shape was performed using custom-built MatLab code (64). Briefly, images of vinculin staining were processed with a median filter followed by subtraction of local background, and interactive intensity threshold was applied to create binary maps of FAs. Standard functions of Image Processing toolbox (MathWorks, Natick, MA) were used to segment the maps and to characterize each segment quantitatively. Measures of eccentricity, size, and number were determined.

Cell adhesion assay. Cell adhesion assays were performed using 96-well cell culture plates. Plates were coated with FN at a concentration of 10 μg/ml for 2 h at 37°C and blocked with 3% BSA for 1 h before plating cells. Cells were plated at a concentration of 2×10^4 cells/well and allowed to attach for 5, 15, 30, and 60 min at 37°C. Following adhesion, nonadherent cells were removed, and adherent cells were fixed with 4% PFA for 15 min at 4°C and stained with 0.5% toloudine blue (Sigma) in 4% PFA for 5 min. After washing three times with water, cells were solubilized with 1% SDS, and the relative absorbance was measured at 595 nm on a microplate spectrophotometer. Each assay was performed in triplicate, and the results averaged.

Cell proliferation assay. The proliferative activity of the cells was determined by 5-bromo-2′-deoxyuridine (BrdU) incorporation using a cell proliferation ELISA kit (Calbiochem, San Diego, CA). A total of 5,000 cells were added to each well of 96-well microplate and incubated for 24 h with PDGF-BB (10 ng/ml). Thereafter, 20 μl of BrdU (1:2,000) were added to each well and incubated for another 2 h. BrdU incorporation was then evaluated by measuring the absorbance at 450 nm, according to the manufacturer's protocols. Each assay was performed in triplicate, and the results averaged.

Cell migration assay. Migration of CFs was assessed using a modified Boyden's chamber method (QCM-FN; Chemicon International, Temecula, CA). Ninety-six hours after infection, CFs were trypsinized and resuspended in DMEM supplemented with 0.1% BSA. The cells were plated into upper chambers of Boyden chambers (2×10^4 cells/chamber) and allowed to migrate for 3 h toward FN or BSA-coated filters on the undersurface of the upper chamber. PDGF-BB (10 ng/ml) was placed in the media of the lower chamber as a chemoattractant. The Boyden chambers were then removed, washed, and stained according to the manufacturer's protocol. Samples were read at 540 nm on a microplate spectrophotometer. Each assay was performed in triplicate, and the results averaged.

Wound-healing assay. Migration of CFs was assessed using a wound-healing assay. CFs were seeded at a concentration of 2×10^5 cells/well onto 60-mm plastic plates in media containing DMEM, 10% BCS, and 1% PS. They were rendered quiescent by their exposure to a defined serum-free DMEM containing 0.1% BSA + 1% PS for 24 h before infection. For virus infection, CFs were incubated with Ad/Cre or Ad/LacZ (200 MOI) for 96 h. Ninety-six hours after infection, CFs were "wounded" by manual scratching with a pipette tip, washed with PBS, photographed in phase-contrast microscope (0 h point), and placed with PDGF-BB (20 ng/ml). Matched pairs were photographed 48 h following PDGF stimulation.

Statistical analysis. All data are expressed as means ± SE. Statistical differences between experimental groups were determined by using Student's *t*-test, and values of $P < 0.05$ were considered significant.

RESULTS

Reduction of FAK expression alters expression of FA proteins but does not change cell adhesion or morphology of CFs. Since no means exist to specifically drive expression of the Cre-recombinase in CFs in the intact heart, we produced FAK-deficient CFs ex vivo. For this, we used Cre-LoxP technology to excise the FAK gene in isolated mouse CFs (3). Cre-recombinase was delivered via adenoviral infection of CFs cultured from homozygous FAK-flox mice ($FAK^{flox/flox}$) (Fig. 1*A*). Cells infected with Ad/LacZ were used as controls throughout the study. Efficiency of the recombinant adenoviral infection of the CFs was first tested using varied MOIs of both Ad/LacZ and Ad/Cre. Evaluation of the cells 48 h following infection at 200 MOI showed that over 80% of the cells were positive for either β-galactosidase or Cre-recombinase expression, by colorimetric or immunofluorescent staining, respectively (data not shown). No cytopathic effects of viral infection were detected at this MOI. Therefore, an MOI of 200 was used for the remainder of the study. We found that Ad/Cre infection caused excision of the FAK gene in the $FAK^{flox/flox}$ CFs, as analyzed by polymerase chain reaction (Fig. 1*B*) and averaged over 70% reduction (69 ± 7% reduction, $P < 0.001$, $n = 5$) of FAK protein in Cre vs. LacZ-infected CFs (see Fig. 3, *A* and *B*). Given this, the Ad/Cre-infected $FAK^{flox/flox}$ CFs were referred to as FAK knockout (KO) cells, while the Ad/LacZ infected $FAK^{flox/flox}$ CFs were used as control cells through the remainder of the study.

Prior studies in cells other than CFs have shown that FAK can modulate cell adhesion (31, 43), formation, and turnover of FAs (19, 24, 37, 48, 52), and also alter cell morphology (52). Therefore, we next examined the effects of FAK deficiency on these properties of CFs. To study FA amount, area, and distribution, we performed immunofluorescence analysis using vinculin as a marker of FAs. FAK-deficient CFs did not show differences in cell morphology. Vinculin immunostaining displayed a similar pattern of distribution in FAK-deficient and control cells, revealing organized patches of FAs at the cellular periphery (Fig. 2). No changes in number, area, or distribution of FAs was detected between control and FAK-deficient cells (data not shown). Paxillin, another marker of FAs, showed the same distribution as vinculin (data not shown). Western blotting was performed to quantitate FA-related protein levels and indicated no change in vinculin, talin, or paxillin expression in the FAK-deficient CFs (Fig. 3, *A* and *B*).

Pyk2 is a cytoplasmic tyrosine kinase that shares approximately a 45% amino acid sequence identity with FAK (33). The expression of Pyk2 in various studies of FAK null cells has been noted to be variable, depending on the cell type, the age of the animal from which the cells were derived, or the culture conditions. For example, Pyk2 expression was enhanced in mouse embryonic fibroblasts (MEFs) from FAK null mice (29, 53), but was not changed in many of the cells or tissue samples derived from conditional FAK KO mice (3, 4, 37). Thus to determine whether Pyk2 levels or function might compensate for FAK deficiency in adult CFs, we examined its expression, activation, and localization in our FAK-deficient cells.

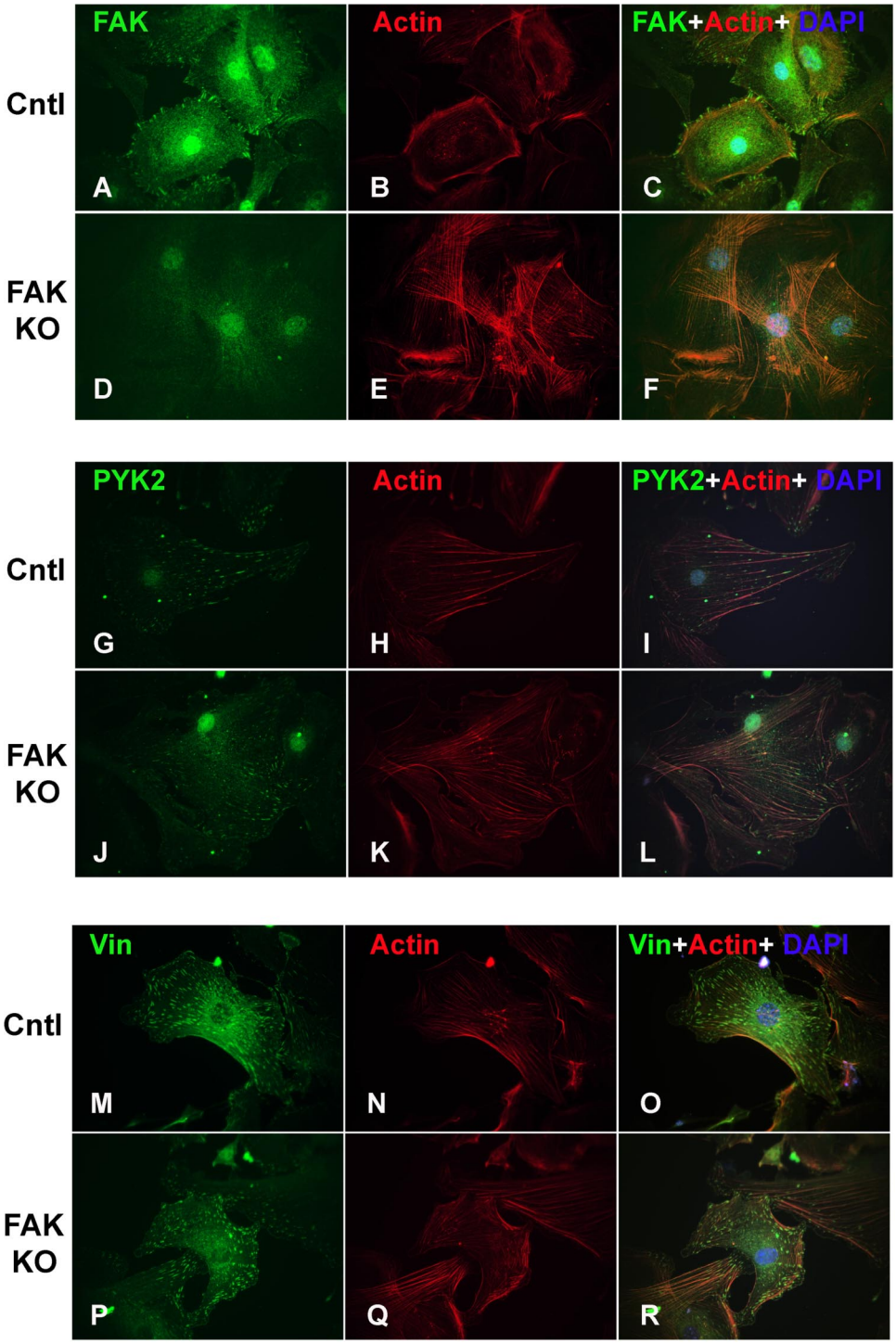


Fig. 2. Localization of FAK, proline-rich tyrosine kinase 2 (Pyk2), and vinculin (Vin) in control (Cntl) and FAK-deficient CFs. FAK (green; *A* and *D*), actin (red; *B* and *E*), and 4,6-diamidino-2-phenylindole (DAPI) (blue; *C* and *F*) are shown in Cntl (*A–C*) and FAK knockout (KO) cells (*D–F*). PYK2 (green; *G* and *J*), actin (red; *H* and *K*), and DAPI (blue; *I* and *L*) are shown in Cntl (*G–I*) and FAK KO cells (*J–L*). Vin (green; *M* and *P*), actin (red; *N* and *Q*), and DAPI (blue; *O* and *R*) are shown in Cntl (*M–O*) and FAK KO cells (*P–R*). Immunofluorescent staining of $FAK^{flox/flox}$ CFs infected with Ad/Cre (FAK KO) or Ad/LacZ (Cntl) showed that FAK protein was localized in focal adhesions (FAs) and nucleus in Cntl cells (*A* and *C*). The amount of FAK was reduced toward background levels in Ad/Cre-infected cells compared with Ad/LacZ-infected cells (*D* and *F*). Pyk2 showed a similar distribution to FAK, localizing in FAs and nucleus in Cntl cells (*G* and *I*), and no changes in Pyk2 localization were seen between Cntl and FAK KO cells (*G* and *I*; *J* and *L*). Vin, a marker of FAs, showed a similar distribution in both Cntl and FAK KO cells (*M* and *O*; *P* and *R*). Phalloidin (red) was used to mark F-actin, and DAPI (blue) labeled nuclei.

By Western blotting, Pyk2 expression was increased in the FAK-deficient CFs compared with control cells (51 ± 16%, $P < 0.05$, $n = 5$; Fig. 3, *A* and *B*). The Pyk2 species detected in FAK-deficient cells migrated slightly slower compared with the one detected in control cells. Phosphorylation of Pyk2 at tyrosine 402 corresponds with its activation. Western blot analyses showed that Pyk2 phosphorylation was also increased in FAK-deficient cells (see Fig. 6, *C* and *D*).

Several studies have indicated that the subcellular distribution of FAK and Pyk2 are different. FAK is most commonly localized in FAs, while Pyk2 is detected in the perinuclear area (53). In adult CFs, FAK was localized at FAs and in the nucleus (Fig. 2). Pyk2 showed the same distribution as FAK (Fig. 2). Furthermore, localization of Pyk2 was similar in the FAK-deficient cells compared with control cells.

Next, we investigated cellular adhesion of the adult mouse CFs to ECM substrates, which are all expressed in the myocardium: laminin, collagen I, and FN. Cell adhesion assays were performed and indicated that wild-type CFs adhered best to FN, regardless of binding time (data not shown). Therefore,

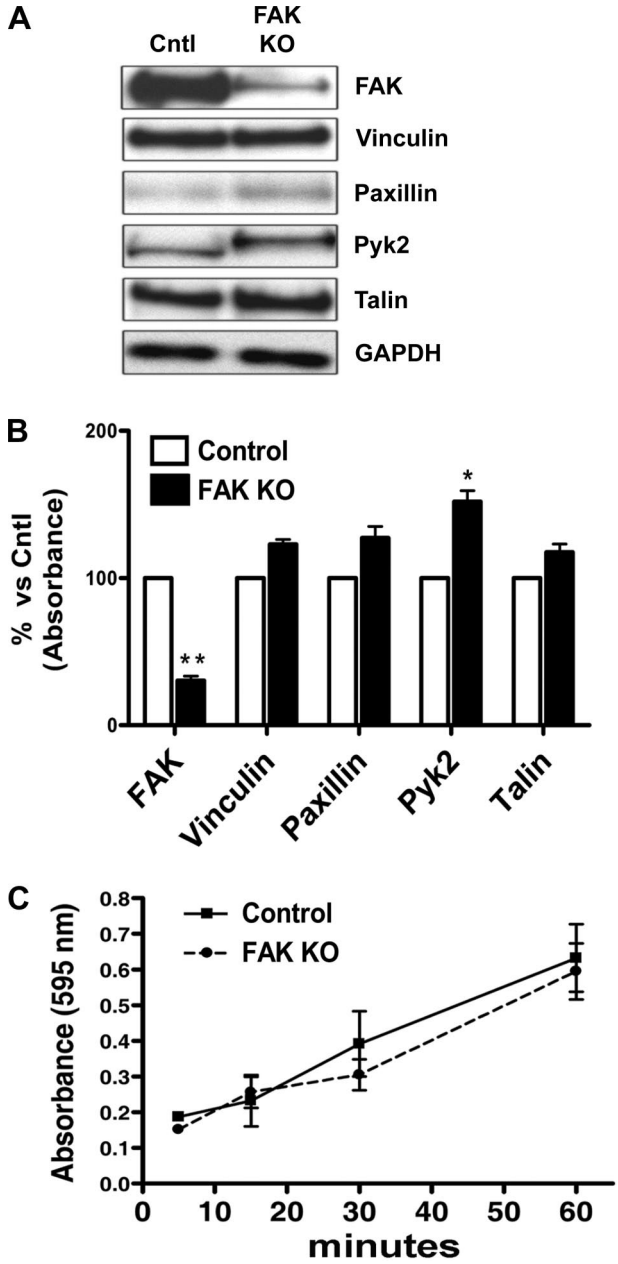


Fig. 3. Reduction of FAK expression alters expression of FA proteins but does not change adhesion to fibronectin of adult CFs. *A*: lysates from $FAK^{flox/flox}$ CFs infected with Ad/Cre or Ad/LacZ (200 MOI) for 96 h were analyzed by Western blotting for FAK and other key FA proteins: Vin, paxillin, Pyk2, and talin. GAPDH was used as a loading Cntl. Blots are representative of 5 independent experiments. *B*: densitometric analysis was performed on Western blots by normalizing FAK, Vin, talin, paxillin, and Pyk2 expression to simultaneously measured GAPDH. CFs infected with Ad/Cre showed a 70% reduction of FAK protein expression (**$P < 0.001$, $n = 5$) and 52% increase of Pyk2 (*$P < 0.05$, $n = 5$) compared with Cntl infected CFs. No other significant alterations in protein expression were found. Data represent mean values ± SE. *C*: adhesion of FAK-deficient CFs (Ad/Cre) to fibronectin was not different from that of Cntl cells when evaluated at 10, 30, and 60 min following plating on a fibronectin matrix.

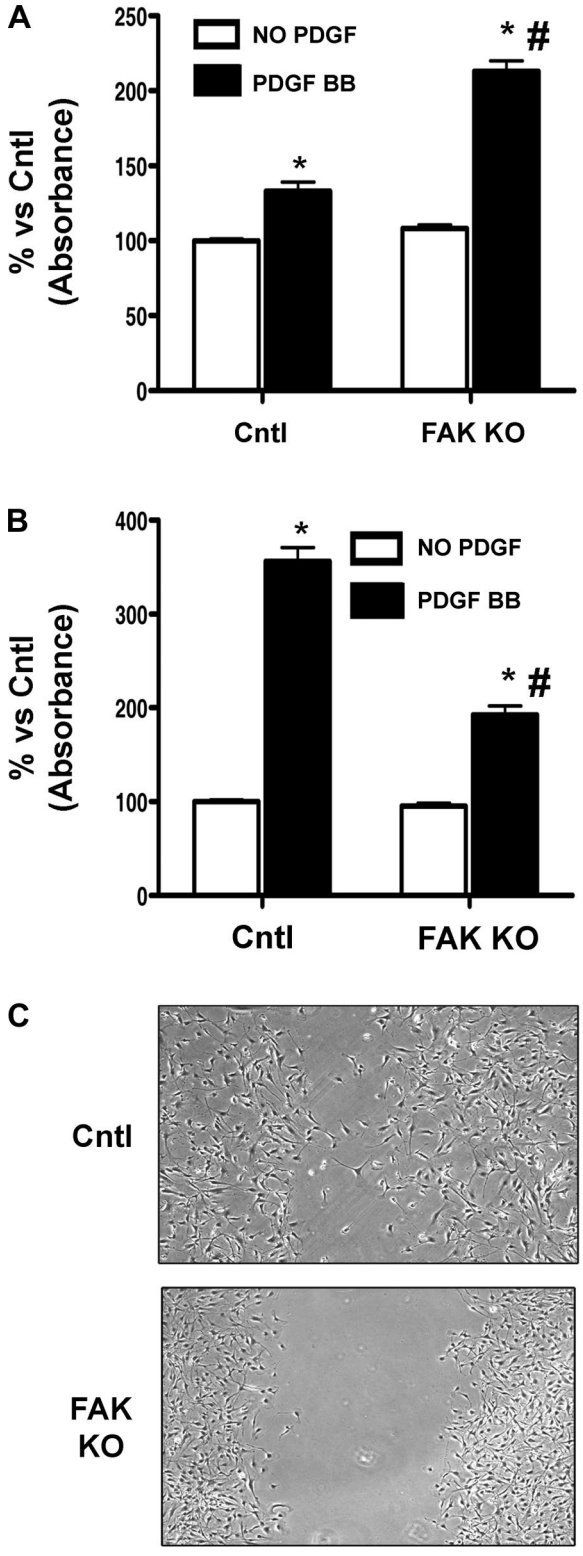
we used FN as a substrate for comparative adhesion of wild-type and FAK-deficient CFs. The FAK-deficient CFs and control-infected cells exhibited no significant differences in adhesion to FN, independent of binding time (5, 15, 30, and 60 min) (Fig. 3*C*). These results show that reduced FAK expression does not affect *1*) the formation and distribution of FAs; *2*) the morphology; or *3*) the adhesive capability of mouse adult CFs to FN, in our culture system. Yet Pyk2 becomes upregulated and activated in CFs deficient in FAK, possibly as a compensatory mechanism.

Proliferation is increased and motility is inhibited in FAK-deficient CFs. FAK has been shown to both positively and negatively influence cell proliferation in a variety of cell types (10, 41). Therefore, we next investigated whether FAK deficiency affected cell proliferation in adult mouse CFs. We used PDGF to provoke cell proliferation, since it has been shown to be a major determinant of this process in a majority of fibroblasts (44) and is a particularly strong mitogen for adult mouse CFs (42). FAK-deficient CFs stimulated with PDGF-BB displayed a significant increase in cell proliferation compared with control cells (FAK KO + PDGF-BB: 213 ± 13%, control + PDGF-BB: 132 ± 12%, $P < 0.0001$, $n = 5$; Fig. 4*A*).

FAK is also known to regulate cell migration through its promotion of stable lamellipodia formation, necessary for directional locomotion, and facilitation of trailing-edge FA release or retraction (4, 23, 37, 48, 58, 60). FAK links both ECM/integrin and growth factor stimulation to intracellular signals promoting cell migration. PDGF-BB is able to stimulate cell migration of CFs. Therefore, we next investigated whether FAK deficiency affects PDGF-BB-stimulated CF cell migration. Migration was first examined using a modified Boyden chamber assay. Migratory activity of the FAK-deficient CFs was reduced compared with control CFs (Ad/Cre + PDGF-BB: 192 ± 16%, Ad/LacZ + PDGF-BB: 356 ± 25%, $P < 0.001$, $n = 3$; Fig. 4*B*). To further examine migration, we also performed companion studies using an in vitro wound-healing assay. When control cells in a confluent monolayer were analyzed for their ability to repopulate the wounded area, they were observed to migrate across the midportion of the open space within 48 h after wounding. In contrast, FAK-deficient CFs migrated slower than control cells (Fig. 4*C*). These results indicate that FAK deficiency disrupts PDGF-BB-stimulated migration of adult mouse CFs.

FRNK inhibits CF motility but not proliferation. Since Pyk2 has also been shown to be involved in cell proliferation and migration (17, 40) and was increased in FAK-deficient CFs, we sought to define if the increase in cell proliferation seen in FAK-deficient CFs was directly related to FAK deficiency, or if it might also be due to increases in Pyk2. For this purpose, we utilized an alternative method to inhibit FAK function using FAK-related nonkinase (FRNK). FRNK is a truncated product of FAK that displaces FAK from the FA and, therefore, acts as an inhibitor of endogenous FAK function. FRNK was overexpressed in normal mouse CFs by infection with a FRNK recombinant adenovirus (39). CFs infected with the Ad/FRNK did not exhibit alterations in FAK expression. Furthermore, Pyk2 expression levels were similar in the Ad/FRNK and control infected cells (Fig. 5, *A* and *B*). CFs treated with Ad/FRNK and also stimulated with PDGF-BB were found to proliferate at the same rate as control cells stimulated with PDGF-BB [Ad/FRNK + PDGF-BB: 116 ± 10%, control (Ad/LacZ) + PDGF-BB: 125 ± 6%, $n = 4$; Fig. 5*C*]. Thus these data suggest that the increased proliferation of CFs deficient in FAK was, at least in part, related to concomitant

upregulation of Pyk2. In contrast to the effects on proliferation, PDGF-BB-stimulated CFs treated with Ad/FRNK migrated less than control cells [Ad/FRNK + PDGF-BB: 102 ± 7%, control (Ad/LacZ) + PDGF-BB: 140 ± 7%, $P < 0.05$, $n = 3$; Fig. 5*D*]. These results indicated that FAK deficiency and not Pyk2 upregulation caused the migration defects seen in FAK-deficient adult mouse CFs.



PDGF-BB-mediated signaling in FAK-deficient CF. Phosphorylation of FAK may occur as a result of diverse cellular stimuli (38) and provoke cellular events, such as migration and proliferation. Whether PDGF-BB causes activation of FAK appears to vary by cell type, with some cells showing robust phosphorylation of FAK on various residues, while other cell types showing no significant change (20, 51, 55). Therefore, we assessed if FAK phosphorylation would occur in mouse CF stimulated with PDGF-BB for 5–30 min. As shown in Fig. 6, *A* and *B*, FAK was highly phosphorylated under basal conditions, and stimulation with PDGF-BB did not cause significant changes in the phosphorylation of FAK at either Tyr 397, its autophosphorylation site, or Tyr 576 (data not shown) in control-infected CFs (Fig. 6, *A* and *B*). With FAK gene excision effected by Cre-recombinase, residual FAK protein was seen to be highly phosphorylated at Tyr 397 (data not shown).

Since we noted basal changes in Pyk2 in the FAK KO cells, we also assessed Pyk2 activation in response to PDGF-BB stimulation. As show in Fig. 6*C*, Pyk2 phosphorylation at Tyr 402, the autophosphorylation site akin to Tyr 397 of FAK, was increased in response to PDGF-BB in control CF. Moreover, PDGF-BB mediated a greater increase in Pyk2 activation in the FAK KO CFs, compared with control cells.

Given our finding that FRNK may modify the migration of the FAK-deficient CFs, as well as prior work that has shown how FRNK overexpression could inhibit phosphorylation of both FAK and Pyk2 (14), we next tested how FRNK might modify the PDGF-BB-mediated Pyk2 activation in our cells. As shown in Fig. 7, FRNK overexpression did not change baseline phosphorylation levels of Pyk2, but inhibited PDGF-BB induction of Pyk2 phosphorylation.

Mitogen-activated protein kinase (MAPK) signaling pathways have been linked to cell proliferation and migration in various cell types, including CFs (18, 28, 32). Furthermore, previous studies have demonstrated that FAK was involved in MAPK activation, which occurs in response to factors such as PDGF-BB (15, 55, 57). Therefore, to further address the molecular mechanisms through which reduced FAK expression inhibited PDGF-BB-stimulated cell motility in CFs, we investigated activation of a series of MAPKs in the FAK-deficient CFs. Adult control and FAK KO CFs were stimulated with PDGF-BB for time points between 5 and 30 min. Whole cell lysates were prepared and analyzed for MAPK activities. The kinetics and activities of PDGF-BB-stimulated ERK42/44 were not altered in the FAK-deficient cells compared with control cells, and JNK and p38 MAPKs were only moderately altered in FAK-deficient cells compared with control cells (Fig. 8). These

Fig. 4. FAK-deficient CFs have reduced migration and increased proliferation in response to platelet-derived growth factor (PDGF)-BB. *A*: cell proliferation was increased in FAK-deficient CFs, as determined by 5-bromo-2′-deoxyuridine (BrdU) incorporation. FAK KO or Cntl CFs were incubated for 24 h with PDGF-BB (10 ng/ml), with the last 2 h in the presence of BrdU. FAK KO CFs showed an increase in proliferation compared with Cntl CFs (#$P < 0.001$ vs. Cntl + PDGF BB; *$P < 0.001$ vs. No PDGF BB, $n = 5$). *B*: migration of FAK-deficient CFs was reduced as quantified using a Boyden chamber migration assay. FAK KO CFs had reduced migration toward 10 ng/ml PDGF-BB in a Boyden chamber assay compared with Cntl CFs when evaluated over 3 h (#$P < 0.001$ vs. Ad/LacZ + PDGF-BB; *$P < 0.001$ vs. No PDGF-BB; $n = 3$). Data represent mean values ± SE. *C*: wound assays show reduced migration of FAK KO CFs compared with Cntl cells when stimulated with 20 ng/ml of PDGF-BB. Matched pairs were photographed 48 h following PDGF stimulation.

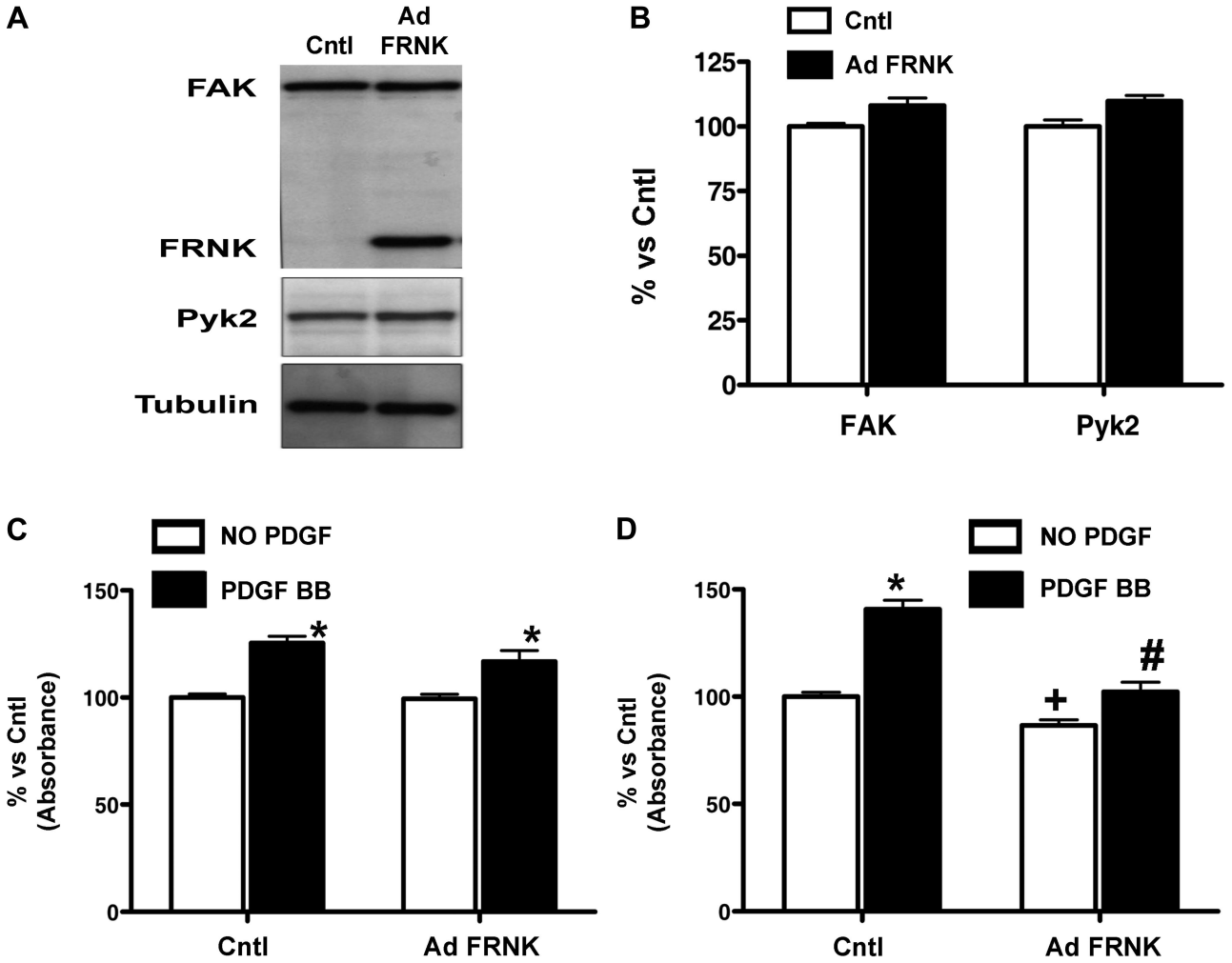


Fig. 5. FAK-related nonkinase (FRNK) expression inhibits PDGF-BB-stimulated migration in CFs but does not change proliferation. *A* and *B*: lysates from CFs infected with Ad/FRNK or Ad/LacZ (Cntl) (25 MOI, each) for 48 h were analyzed by Western blotting (*A*) with antibodies to FAK, Pyk2, and tubulin. The blots are representative of 3 independent experiments. *B*: densitometric analysis was performed by normalizing FAK and Pyk2 expression to simultaneously measured tubulin. Data represent mean values ± SE. *C*: cell proliferation of CFs was not altered by FRNK as determined by BrdU incorporation. CFs infected with Ad/FRNK or Ad/LacZ (Cntl) were incubated for 24 h with 10 ng/ml PDGF-BB with the last 2 h in the presence of BrdU. CFs infected with Ad/FRNK did not show any difference in proliferation compared with Cntl CFs (*$P < 0.001$ vs. No PDGF-BB, $n = 4$). *D*: FRNK causes reduced migration of CFs when quantified using a Boyden chamber migration assay. CFs infected with FRNK (Ad/FRNK) or LacZ (Cntl) were allowed to migrate for 3 h toward 10 ng/ml PDGF-BB. Increased expression of FRNK in CFs caused a reduction in migration compared with Cntl CFs (#$P < 0.01$ vs. Cntl + PDGF-BB, *$P < 0.01$ vs. No PDGF BB, $^{+}P < 0.05$ vs. Cntl, $n = 3$). Data represent mean values ± SE.

results indicate that MAPK activation is preserved in adherent, FAK-deficient CFs stimulated with PDGF-BB.

DISCUSSION

Proliferation and migration of CFs are key events in cardiac remodeling. Excessive proliferation and migration, together with matrix production and deposition, can lead to fibrosis, which increases myocardial stiffness and may lead to diastolic and potentially systolic dysfunction. Many factors have been identified that regulate CF growth and migration, such as growth factors, cytokines, or mechanical stress (2, 7). While it is clear that the responses to these factors can be dependent on ECM/integrin interactions, the specific role of FAK, one of the main kinases that transmits integrin signaling, is not fully understood.

In this study, we generated FAK-deficient cells from fully mature CFs by Cre-mediated excision of a floxed-FAK allele and showed that these cells had no changes in FA or gross cellular morphology, nor expression levels of the FA and adapter proteins vinculin, talin, or paxillin. Paralleling the reduction in FAK, we observed an increase in Pyk2 expression and activity, likely as a compensatory mechanism for loss of FAK. With FAK reduction, the adult CFs still had adhesive properties equivalent to control cells, but showed decreased migration and increased proliferation in response to PDGF-BB. Companion studies with FRNK as an inhibitor of FAK demonstrated that FAK plays a key role in PDGF-BB-induced migration in mouse adult CFs. Finally, while PDGF-BB caused an increase of Pyk2 phosphorylation in the FAK-deficient CF, there was no change in ERK activation, and only minimal changes in activity of p38 and JNK.

Cell adhesion to ECM is essential for processes such as cell cycle progression and to protect cells from apoptosis (21). In migratory cells like fibroblasts, motility occurs by the forma-

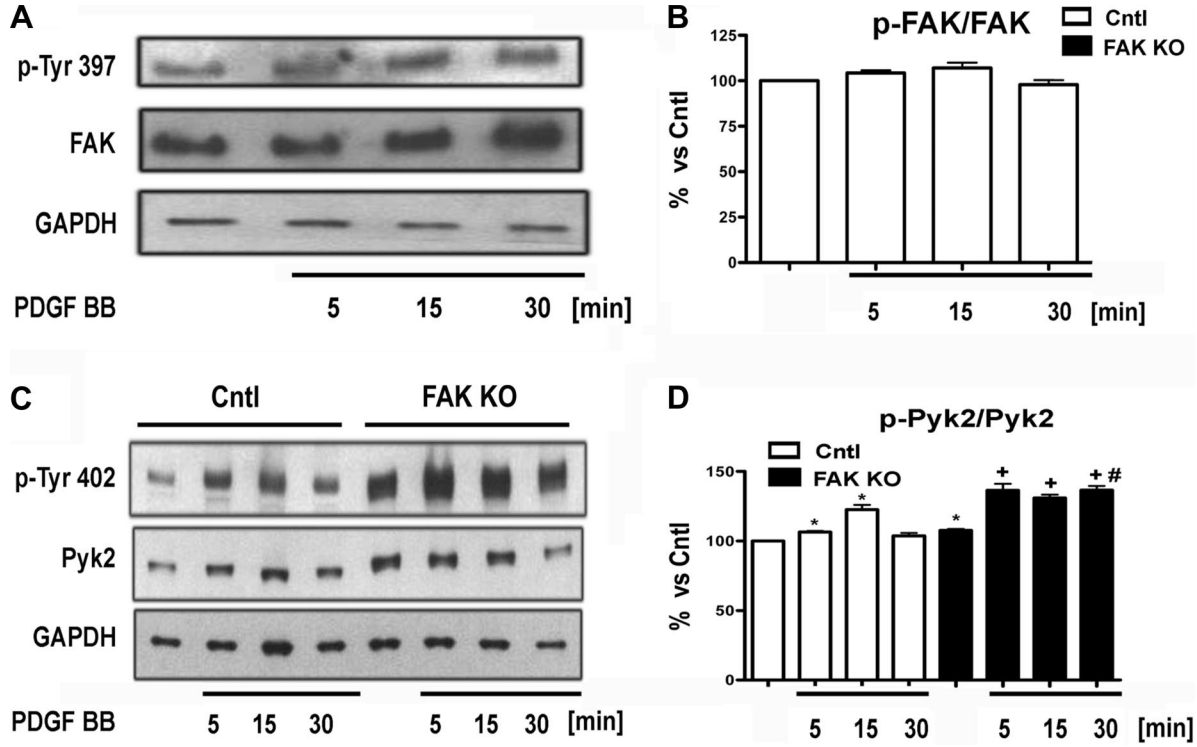


Fig. 6. FAK and Pyk2 activation in response to PDGF-BB. FAK KO or Cntl CFs were treated with 10 ng/ml PDGF-BB for various times as indicated. *A*: total and Tyr 397 phosphorylated FAK (p-FAK) protein were analyzed by Western blotting and showed no increase in phosphorylation in Cntl cells after PDGF-BB treatment. *B*: densitometric analysis was performed on Western blots by normalizing p-FAK (Tyr 397) to total FAK ($n = 3$). Data represent mean values ± SE. *C*: total and Tyr 402 phosphorylated Pyk2 (p-Pyk2) protein were analyzed by Western blotting and showed an increase in phosphorylation at Tyr 402 in Cntl and FAK KO cells in response to PDGF-BB. *D*: densitometric analysis was performed on Western blots by normalizing p-Pyk2 (Tyr 402) to total Pyk2 [$^{*}P <$ 0.05 vs. Cntl (No PDGF-BB), $^{+}P < 0.01$ vs. FAK KO (No PDGF-BB), $^{\#}P < 0.01$ vs. Cntl+ PDGF-BB (30 min), $n = 7$]. Data represent mean values ± SE.

tion of new FAs at the leading edge of the cell. In our study, FAK-deficient CFs exhibited no significant alteration in adhesion to FN. Studies of FAK null embryonic stem cells, as well as keratinocytes, have similarly shown no changes in surface levels of integrins and adhesion to several ECM substrates (22, 48). Thus it appears that FAK is not necessary for normal adhesion of adult mouse CFs.

Several publications that focus on the role of FAK in FA assembly and turnover have utilized undifferentiated embryonic cells such as MEFs. Early studies found that these cells have an increase in FA size and number, as well as reduced migration, and linked these changes to a decrease in FA turnover (35). More recently, Lim and colleagues (29) demonstrated that these alterations in FAs were caused by upregulation of Pyk2 in the FAK-deficient cells, as reduction of Pyk2 expression in the $FAK^{-/-}$ MEFs resulted in normalization of both the number and size of FAs. Still, additional studies underscore the necessity to research the role of FAK in differ-

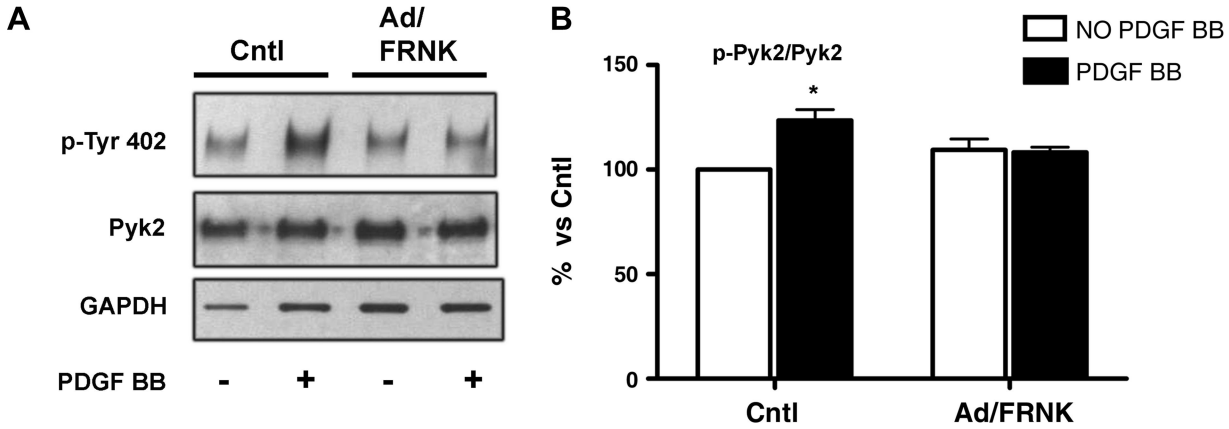


Fig. 7. Pyk2 activation in response to PDGF-BB in FRNK-treated cells. Ad/FRNK or LacZ (Cntl) CFs were treated with 10 ng/ml PDGF-BB for 30 min. *A*: total and Tyr 402 p-Pyk2 protein were analyzed by Western blotting and showed no increase in phosphorylation in Ad/FRNK-treated cells after PDGF-BB treatment. *B*: densitometric analysis was performed on Western blots by normalizing p-Pyk2 (Tyr 402) to total Pyk2 ($n = 5$). $^{*}P < 0.05$ vs. Cntl (No PDGF-BB). Data represent mean values ± SE.

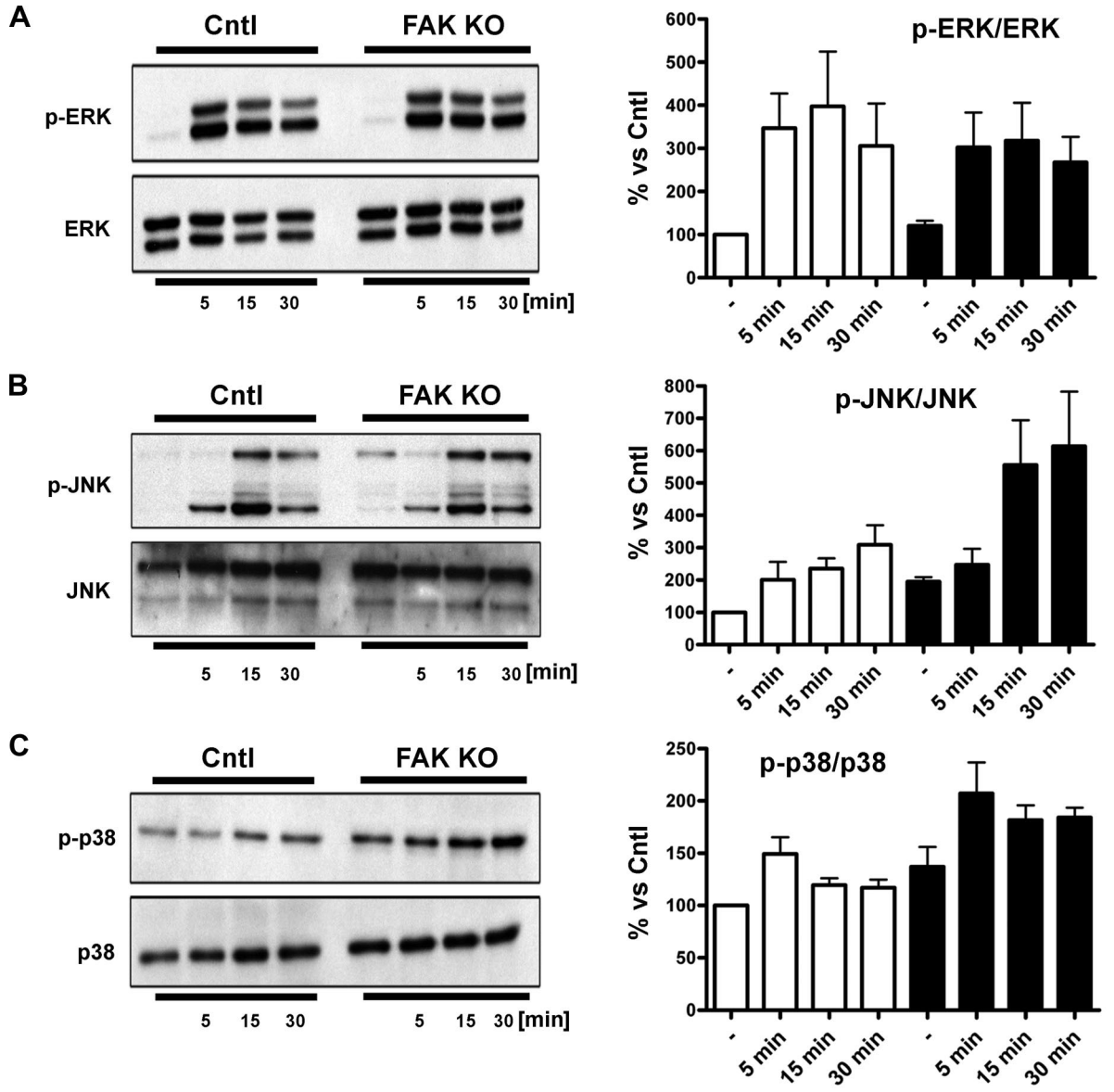


Fig. 8. Activation of MAPKs by PDGF-BB in FAK-deficient CFs. FAK KO or Cntl CFs were treated with 10 ng/ml PDGF-BB for various times as indicated. Activated and total ERK1/2 (*A*), JNK (*B*), and p-38 (*C*) were analyzed by Western blotting and showed only a trend toward increased phosphorylation of JNK and p38 in the FAK-deficient CFs compared with Cntl cells. Overall, no statistically significant differences in MAPK activation was noted in the KO cells. The blots are representative of at least three independent experiments.

entiated cell types, as we have done here using adult CFs. For example, FAK-deficient keratinocytes had a decreased rate of FA disassembly leading to abnormally large FAs (48). In endothelial and intestinal epithelial cells, FAK deficiency caused a decrease in the number of FAs (4, 24), perhaps due to a decrease in FA assembly, while FAK null macrophages showed reduced assembly and disassembly rates of FAs, leading to preservation of FA number and size (37). In the present study, no change in FA number or distribution was found. Likewise, protein levels of the FA proteins vinculin, talin, and paxillin were not altered, suggesting that the assembly and disassembly rates of FA were not significantly altered in FAK-deficient cells derived from adult CFs.

FAK-deficient MEFs have an altered and rounded epithelial-like morphology (3, 37, 48, 58, 62). Recent work demonstrates that this morphological change appears to be caused by the increased levels of Pyk2 found in the FAK-deficient MEF, leading in turn to an increase in p190RhoGEF expression and aberrant RhoA activation (29). Indeed, when we made adult mouse CFs deficient in FAK, upregulation and activation of Pyk2 occurred, but the CFs did not show any change in cell morphology compared with control cells. These differences indicate that the concomitant FAK deficiency and Pyk2 upregulation likely have unique effects in differentiated fibroblasts derived from adult tissue, as we studied here, vs. an immature embryonic source. Furthermore, these data are in line with other studies that showed that fibroblasts from different organs display distinct and characteristic transcriptional patterns and should be analyzed as distinct cell types, despite all being of a "fibroblast" lineage (8, 50). Furthermore, the ex-

pression and subcellular localization of Pyk2 appear distinct in CFs in contrast to MEFs. MEFs have minimal Pyk2 expression, while wild-type CFs display significant Pyk2 protein levels. On the other hand, MEFs display Pyk2 in a perinuclear pattern (53), while CFs show Pyk2 to be localized to FA and nucleus. This FA localization of Pyk2 in CFs has been also shown in other cell types, such as keratinocytes (48), smooth muscle cells (5), and osteoblasts (25). These data suggest unique roles of Pyk2, depending on cell type, and also indicate that FAK and Pyk2 expression are finely balanced. Thus when FAK is decreased, Pyk2 may be increased and/or activated. Finally, our data showed not only how the FAK-deficient CFs exhibited increased Pyk2 protein levels as well as an activation, but that the species of Pyk2 that increased was of a higher molecular weight than that detected in control cells. Pyk2 of a higher molecular weight was also detected in fibroblasts derived from embryos of FAK KO mice (53). This may result from an increase in the phosphorylation status of Pyk2 or, as Sieg et al. suggest (53), to a change in the protein variant/isoform of Pyk2, which becomes expressed in these cells. Further studies will be necessary to answer this question.

Role of FAK in proliferation. Many studies support the important role that FAK plays in regulating cell cycle progression by integrating signals from integrins and growth factor receptors (38). Previous studies have shown that inhibition of FAK expression results in decreased cell proliferation. Gilmore and Romer (13) used human umbilical vein endothelial cells and 3T3 cells to demonstrate that the displacement of the endogenous FAK by overexpression of a construct containing a FAK FA targeting sequence, but not the kinase domain, decreased cell proliferation. Zhao et al. (65) also demonstrated in 3T3 cells that inhibition of FAK provoked a decrease of cyclin D1 expression, a regulator of cell progression through the G1 to S phase. Subsequent studies in smooth muscle, endothelial, and hepatic cells (43, 56) support the role of FAK as a positive regulator of cell proliferation. Our results showed that FAK protein depletion in CFs increased PDGF-BB-induced proliferation. The CFs were found to have upregulation and activation of Pyk2, in parallel with the reduction of FAK. Thus we carried out companion studies to inhibit FAK activity by overexpression of FRNK. In this case, Pyk2 levels were not changed, and cell proliferation was normal. Thus it is likely that the proliferative changes of FAK null CFs may be at least in part linked to the changes in Pyk2.

In agreement with this result was a recent study that affected upregulation of Pyk2 in FAK KO MEFs, leading to proliferation through the upregulation of p190RhoGEF expression (29).

Role of FAK in migration. FAK has been shown to play a key role in cell motility in a range of cell types, including embryonic fibroblasts, smooth muscle cells, endothelial cells, epithelial cells, 3T3 cells, adenocarcinoma cells, macrophages, and keratinocytes (13, 15, 16, 24, 29, 37, 48, 49, 51, 56). In these cells, the function of FAK was investigated by several methods, including use of inhibitors, such as FRNK or FAK truncated products, or by producing FAK deficiency invoked by global KO, small interfering RNA, or a Cre/LoxP system. These studies showed that inhibition of FAK function or expression lead to a decrease in the migratory responses to growth factors like PDGF-BB and VEGF, G-protein-coupled receptor agonists like lysophosphatidic acid, and induction by ECM proteins such as FN. In contrast, a study using HeLa cells plated on a collagen substrate showed that cell migration was enhanced by inhibition of FAK expression or activity through the dissolution of *N*-cadherin-mediated cell-cell contacts (62). Our studies in adult mouse CFs are in agreement with the majority of studies, in that FAK depletion or inhibition decreased migration. As PDGF-BB increased migration of the FAK-deficient CFs, this suggests that a component of PDGF-BB-mediated migration is independent of FAK activity. It is possible that this residual migratory effect of PDGF-BB could be provoked through Pyk2, as several studies in different cells types, such as macrophages (36) and endothelial cells (27), have linked Pyk2 with cell migration. In agreement with this hypothesis is that small hairpin RNA-mediated inhibition of Pyk2 in FAK KO MEFs caused reduction of residual motility on FN. Still, the upregulation of Pyk2 in the FAK-deficient CF background was unable to completely compensate/rescue the motility defects of these cells, underscoring the essential role of FAK in migration of CFs. Another possibility that we cannot rule out is that residual FAK could still be functional and responsible for the residual migration after PDGF-BB treatment.

FAK is known to regulate cell migration through its promotion of stable lamellipodia formation, necessary for directional locomotion, and facilitation of trailing-edge FA release or retraction (4, 15, 23, 37, 48, 58, 60). Additional work will be needed to elucidate if FAK is acting through these mechanisms in mouse CFs.

PDGF-BB-mediated signaling in FAK-deficient CF. In the CFs studied here, FAK was highly phosphorylated at Tyr 397 in basal conditions where the cells were attached, and subsequent PDGF-BB stimulation did not cause significant changes in phosphorylation at either Tyr 397 or Tyr 576 (data not shown). Prior work in agreement with our finding has showed that PDGF-BB does not increase Tyr 397 phosphorylation, but produced alteration in FAK localization, as it became associated with an activated PDGF-receptor complex as cell migration was induced by PDGF-BB (51). Although many studies have shown that phosphorylation of FAK at Tyr 397 increases upon cell stimulation with a variety of factors, a growing number has also shown that the basal phosphorylation levels of this tyrosine is high in cells attached to a substratum, and that subsequent treatment with stimuli does not enhance its phosphorylation further (16, 51). In contrast, the residual FAK protein assayed in pools of the FAK-deficient CFs was highly phosphorylated at Tyr 397. This was previously reported in human carcinoma cells treated with antisense oligonucleotides to reduced FAK (16). The residual FAK protein in cells within a group of cells may still be functional and thus contribute to PDGF-BB signaling involved in proliferation and migration.

As opposed to FAK, we found that Pyk2 became phosphorylated at Tyr 402 in response to PDGF-BB stimulation, and that this phosphorylation was higher in FAK-deficient cells. This further underscores the compensatory role of Pyk2 in FAK-deficient cells and also suggests that Pyk2 could be playing a role in the increase of proliferation shown by FAK-deficient cells after PDGF-BB treatment.

MAPK signaling in FAK-deficient cells. MAPKs are a family of serine/threonine kinases that are well known to play an important role in cell proliferation and migration (18). Previous studies have demonstrated that FAK was involved in MAPK activation, which occurs in response to factors such as

PDGF-BB (15, 55, 57). Our results show that the kinetics of PDGF-BB-stimulated ERK1/2, JNK, and p38 activities were not significantly altered in FAK-deficient cells compared with control cells. In contrast with our results, previous studies in p53-transformed FAK null MEFs show a reduction in the extent and duration of integrin and growth factor-stimulated ERK, as well as JNK activation (26). In others studies with vascular smooth muscle cells, FRNK overexpression caused inhibition of the duration and extent of ERK activation by PDGF-BB. Furthermore, these investigators directly showed that ERK activity was required for PDGF-BB-induced migration (15). In other studies with vascular smooth muscle cells, FRNK expression inhibited PDGF-BB activation of JNK (55). In carcinoma cells, FRNK expression blocked ERK2 and JNK activation, as well as the ERK-dependent, EGF-stimulated migration (16). Differences between our own data and this prior work display the distinct functional role that FAK has in specific cell types.

Conclusion. In conclusion, we used Cre LoxP technology to excise the FAK gene specifically in adult CFs. With this, we showed that FAK played critical roles in PDGF-BB-induced migration. FAK deficiency increased expression of Pyk2 but did not significantly change activities of ERK, JNK, or p38 MAPKs. Altered migration of CFs may occur in a variety of heart diseases, such as that found in the remodeling response following myocardial infarction. Improved knowledge of the mechanisms involved in this process may lead to new therapeutic targets, which could mitigate detrimental cardiac fibrosis. Further studies using in vivo models will be necessary to directly assess the role of CF FAK in a range of myocardial disease processes.

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